

PREPARING MEMBRANE RECEPTORS FROM EXTRACELLULAR
BACILOVIRUSES

The invention relates to the production of
5 membrane receptors in a baculovirus/insect cell system.

Over the past few years, heterologous
expression systems have often been used to study the
expression as well as the pharmacological and
biochemical characteristics of membrane receptors.

10 Although a significant expression can be
obtained in some expression systems in mammalian cells,
there have been problems, in particular in the case of
some types of receptors such as the G-protein-coupled
receptors.

15 The G-protein-coupled receptors belong to the
superfamily of receptors with seven transmembrane
domains. They comprise, for example, the adrenergic or
muscarinic receptors, and all have the same structure
which is made up of a polypeptide chain comprising
20 seven hydrophobic domains which cross the membrane
lipid bilayer.

When it is desired to express these receptors
in mammalian cell systems, a relatively low density of
receptors expressed by the said cells, rarely exceeding
25 a few picomoles of receptor per milligram of membrane
protein, is generally obtained. Although these levels
of expression are sufficient for a functional and
pharmacological characterization, they clearly limit
the type of biochemical, biophysical and structural
30 studies which can be carried out. A fortiori, this
expression system cannot be used for the production of
receptors in a large quantity, for example for their
therapeutic use.

To increase the quantity of receptors obtained,
35 various teams have sought to produce them in a
baculovirus/insect cell system; in many cases,
baculoviruses expressing G-protein-coupled receptors
have been able to produce these recombinant receptors
in cells of the *Spodoptera frugiperda* Sf9 or Sf21

lines, up to levels reaching 30 to 100 picomoles per milligram of membrane protein. These systems have made it possible to make significant progress in the study of the palmitoylation of receptors and also to study
5 the effects produced by various agonists and antagonists, or to carry out the reconstitution of artificial receptors.

However, the baculovirus/insect cell system has the major disadvantage of expressing a high proportion
10 of inactive receptors. The receptors, which are recovered in the membrane fraction of the cells infected with the baculoviruses are in an immature and incompletely glycosylated form. This probably results from a saturation of the normal post-translational
15 maturation pathway, which brings about the retention of immature receptors in the membranes of the endoplasmic reticulum or in the Golgi apparatus. To obtain functional receptors, it is necessary in this case to include a purification step based on the biological
20 activity of the receptor (for example an affinity chromatography step).

It would therefore be necessary to develop a system which makes it possible to easily separate the plasma membrane comprising the mature receptors from
25 the other membrane fractions such as the endoplasmic reticulum or the membranes of the Golgi apparatus which comprise the biologically inactive, immature receptor.

It has recently been shown that the infection of Sf9 cells with a baculovirus encoding the HIV1 Gag gene (Pr55 Gag) brings about the budding of particles
30 carrying the Gag protein (Gag particles) which are released into the extracellular medium. It has been suggested that these Gag particles carry with them, during their budding, the plasma membrane and the
35 proteins associated with it.

The inventors have formulated the hypothesis that the coexpression, in a baculovirus/insect cell system, of a G-protein-coupled receptor and of Pr55 Gag can promote the release of the Gag particles expressing

only the mature receptors which are correctly inserted into the plasma membrane. To test this hypothesis, the inventors infected Sf9 cells with baculoviruses encoding the human adrenergic receptor β 2AR and the Pr55 Gag protein. Surprisingly, they then observed that the β 2AR receptor is almost completely absent from the Gag particles, but is, on the other hand, present at a high density in extracellular baculovirus particles. In addition, the receptors expressed in these extracellular baculoviruses are correctly glycosylated and normally active.

The present invention relates to the use of these extracellular baculoviruses for the production of preparations of a membrane receptor.

The subject of the present invention is a method of producing a recombinant membrane receptor in a baculovirus/insect cell system, from a culture of insect cells infected with a recombinant baculovirus expressing the gene encoding the said membrane receptor, which method is characterized in that the said membrane receptor is obtained from extracellular baculoviruses produced by the said infected cells.

According to a preferred embodiment of the present invention, the said receptor belongs to the superfamily of receptors with seven transmembrane domains; this is for example a receptor of the family of G-protein-coupled receptors.

Recombinant baculoviruses expressing the gene encoding the membrane receptor which it is desired to produce are obtained by cloning the said gene under transcriptional control of an appropriate promoter of the said baculovirus, according to methods well known per se to persons skilled in the art.

Any strong baculovirus promoter which can be used for the expression of heterologous genes, such as for example the polyhedrin promoter (*polh*) or that of the P10 protein, may be used for the production of a recombinant baculovirus which can be used within the framework of the present invention.

According to a preferred embodiment of the method in accordance with the invention, it comprises a step during which the extracellular baculoviruses produced by the said infected cells are harvested and they are separated from the cellular fractions. The harvesting and the separation of the extracellular baculoviruses may be carried out by successive centrifugations, for example in the following manner: a first centrifugation is carried out at about 500xg, at the end of which the supernatant containing the extracellular baculoviruses is collected. This supernatant is subjected to a centrifugation at about 45,000xg; the resulting pellet which contains the extracellular baculoviruses is resuspended, and the suspension is subjected to a centrifugation at about 500xg; the supernatant resulting from this centrifugation is centrifuged at about 45,000xg, and the pellet, which contains the extracellular baculoviruses, is recovered. Advantageously, the extracellular baculoviruses may also be purified by sucrose gradient centrifugation, or any other equivalent method.

According to another preferred embodiment of the method in accordance with the invention, it comprises a step during which the extracellular baculoviruses produced by the said infected cells are lysed; advantageously, it also comprises a step during which the lysate obtained at the end of the preceding step is fractionated, and the fraction comprising the said membrane receptor is recovered.

The purified preparations and the lysates of extracellular baculoviruses, as well as their fractions comprising the membrane receptor, which are capable of being obtained by the methods defined above constitute membrane receptor preparations which also form part of the subject of the present invention. These preparations consist of active and fully mature receptors, unlike the membrane receptor preparations obtained in the prior art from the plasma membranes of

infected cells, which comprise a high proportion of inactive receptors, and which can only be used after an additional step of purification on the basis of the activity of the relevant receptors, for example after
5 affinity chromatography.

By contrast, the membrane receptor preparations in accordance with the invention are characterized in that, prior to any purification carried out on the basis of the activity of the relevant receptor, at
10 least 90%, and preferably at least 95%, of the said receptor is in an active form.

Preparations, in accordance with the invention, of a membrane receptor may be used to prepare the said receptor in a purified form, with a much better yield
15 than that which could be achieved from membrane receptor preparations obtained in the prior art from the plasma membranes of infected cells.

The membrane receptor preparations in accordance with the invention, as well as the
20 extracellular baculoviruses obtained using the method in accordance with the invention, may also be used directly, for example as a system for studying the properties of membrane receptors, as a system for screening molecules which are active on these membrane
25 receptors, or alternatively for studying their post-translational modifications such as phosphorylation or palmitoylation.

The present invention will be understood more clearly with the aid of the additional description
30 which follows, which refers to exemplary embodiments of the method in accordance with the invention for the preparation of membrane receptors.

EXAMPLE 1: PREPARATION OF RECOMBINANT BACULOVIRUSES 35 EXPRESSING A G-PROTEIN-COUPLED RECEPTOR

A recombinant baculovirus expressing $\beta 2AR$ is obtained by cloning a DNA sequence consisting of the cDNA for $\beta 2AR$ in fusion with the c-myc epitope, obtained as described by MOUILLAC et al. [J. Biol.

Chem., 267, 21733-21737 (1992)], at the NheI site of the transfection/recombination vector pJVNheI (marketed by the company INVITROGEN). This vector was transfected with the linearized genome of a baculovirus AcMNPV (sold by the company INVITROGEN) into Sf9 cells; the recombinant baculovirus obtained in this manner is called c-myc- β 2AR.

In the same manner, the sequence encoding the muscarinic receptor M1 and the sequence encoding the dopaminergic receptor D1 [respectively described by ALLARD et al. Nucleic Acid Research, 15, p 10604, (1987) and by DEARRY et al., Nature, 347, p 72, (1990)] were cloned in order to obtain the recombinant baculoviruses (respectively called M1-R and D1-R) expressing these receptors.

a) Culture and infection of the cells, and harvesting of the extracellular baculoviruses:

Sf9 cells are cultured at 27°C in culture flasks in a 100 ml suspension (BELLCO GLASS) in supplemented GRACE medium (GIBCO) containing 10% foetal calf serum (FCS), and 0.001% pluronic acid. 60 ml of suspension of cells (2×10^6 /ml) are infected with the recombinant baculovirus expressing β 2AR, D1 or M1, at a multiplicity of infection varying between 2 and 5.

The cells are harvested by centrifugation at 500xg for 5 min at 4°C.

The viral particles are isolated after harvesting the cells, by centrifugation of the culture supernatant at 45,000xg for 20 min at 4°C. The pellets obtained are resuspended at 4°C in a volume of phosphate-buffered saline (PBS) equal to 1/10th of the volume of the initial culture, and centrifuged at 500xg for 5 minutes at 4°C; the supernatant for this centrifugation at 500xg is again centrifuged at 45,000xg for 20 min at 4°C.

b) Purification of the baculovirus particles on a sucrose gradient

The pellet of viral particles which is obtained from 100 ml of cultures of Sf9 cells infected with the

recombinant baculovirus expressing β 2AR, M1 or D1 is resuspended in 1.2 ml of TE solution (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), in the presence of protease inhibitors).

5 The suspension is deposited at the top of a tube containing a linear gradient (25%-56%) of sucrose in TE solution. The tubes are centrifuged at 100,000 \times g for 90 minutes. The gradient is collected from the top to the bottom of the tube, in 20 fractions. The first
10 fraction has a volume of 1.4 ml, and the other 19 are 500 μ l.

EXAMPLE 2: DEMONSTRATION OF THE β 2AR ACTIVITY IN THE EXTRACELLULAR RECOMBINANT BACULOVIRUSES

15 The Sf9 cells infected with the recombinant baculovirus expressing β 2AR are cultured, and the baculoviruses are harvested as described in Example 1 a), 24 hours, 48 hours, 72 hours, 96 hours and 120 hours after the infection.

20 The activity of the β 2AR receptors is evaluated by measurements of saturation and of competitive binding which are carried out as described by BOUVIER et al. [Mol. Pharmacol. 33 :133-139 (1982)] using [125 I]iodocyanopindolol ([125 I]ICYP) as labelled ligand.

25 The pellets of viral particles which are obtained are resuspended at 4°C in the buffer which will be used for the reaction. Aliquots of this suspension of viral particles, corresponding to 0.2 to 1 μ g of proteins are mixed with 5 to 350 pM of
30 radioligand [125 I]ICYP in a final volume of 500 μ l. The non-specific binding is evaluated using 10 μ M of alprenolol.

 Under these conditions, the β 2AR activity is detected in the cell culture supernatants from 48 hours
35 after the infection, reaches its maximum 72 hours after the infection, and remains constant up to 120 hours after the infection.

 These results are illustrated in Figure 1.

This figure also shows the results obtained, under the same experimental conditions, on culture supernatants of cells infected both with the recombinant baculovirus c-myc- β 2AR, and a recombinant baculovirus expressing the HIV Gag protein (\bullet = β 2AR; \diamond = β 2AR + Gag). It is observed that, contrary to what was initially expected, the presence of the Gag protein does not increase the quantity of β 2AR in the culture supernatants.

It is also observed that the β 2AR activity detected in the supernatants does not come from the cell lysis, since this activity appears 48 hours after the infection; that is to say at a time where the majority of the infected cells are still viable, and does not increase between 72 and 120 hours after the infection, in spite of the substantial cell lysis which occurs at this time.

The nature of the particles in the supernatant carrying the β 2AR activity was checked by electron microscopy after labelling these particles with the aid of an antibody directed against the c-myc antigen, or an antibody directed against the β 2AR receptor. It was thus observed that the particles recognized by either of these antibodies are 15×100 nm rods, which corresponds to extracellular baculoviruses.

In the case of the co-infection with a baculovirus expressing the Gag protein, the presence of particles exhibiting the morphology of the Gag particles, and which are recognized by an anti-Gag antibody, is observed, in addition, in the supernatant; however, unlike the extracellular baculoviruses, these Gag particles are only very weakly recognized by the anti-c-myc and anti β 2AR antibodies.

The presence of the β 2AR receptor was also verified in the preparations of recombinant baculovirus c-myc- β 2AR which are purified on a sucrose gradient, as described in Example 1 b) above.

The β 2AR activity was determined according to the protocol described in Example 2 above, on various fractions of the gradient.

In parallel, the detection of the vp80, gp67 and vp39 antigens of the baculovirus AcMNPV, using a polyclonal antibody directed against these antigens, was carried out on the same fractions. The results obtained show that the β 2AR activity cosediments with the viral particles.

All the results obtained above show that not only are molecules of the receptor expressed in the recombinant extracellular baculoviruses, but also that they are active molecules.

The quantification of the β 2AR activity in the recombinant extracellular baculovirus preparations purified on a sucrose gradient makes it possible to evaluate the density of the active receptor at about 25 pmol/mg of total proteins.

EXAMPLE 3: COMPARISON OF THE FORMS OF THE β 2AR RECEPTOR WHICH ARE PRESENT IN CELL MEMBRANE PREPARATIONS AND IN THE EXTRACELLULAR BACULOVIRUSES

Sf9 cells infected with the recombinant baculovirus c-myc- β 2AR are harvested 72 hours after the infection. The extracellular baculoviruses c-myc- β 2AR are harvested from the culture supernatant of these cells, and the viral particles are purified as described in Example 1 b).

The membranes of the Sf9 cells are prepared as follows: the cells are centrifuged at 500xg for 5 minutes at 4°C, rinsed once with PBS buffer at 4°C, and resuspended in lysis buffer (20 mM Tris-HCl, 5 mM EDTA, pH 7.4 containing 5 µg/ml leupeptin, 5 µg/ml of trypsin inhibitor and 10 µg/ml of benzamidine) at 4°C. The cells are then lysed by sonication, the lysates are centrifuged for 5 min at 500xg at 4°C and the supernatants centrifuged at 45,000xg for 20 min at 4°C. The pellets are resuspended at 4°C in reaction buffer

(75 mM Tris-HCl (pH 7.4), 12.5 mM magnesium chloride, 2 mM EDTA), in the presence of protease inhibitors.

6 mg of the cell membrane preparation or of the purified baculovirus preparation are added to 5 ml of solubilization buffer (10 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4, 0.3% n-dodecyl maltoside (BOEHRINGER MANNHEIM) in the presence of protease inhibitors. The solubilization is carried out for 90 min at 4°C.

The solubilized receptors are purified by affinity chromatography as described below. The ALPRENOLOL-SEPHAROSE affinity matrix is synthesized according to the method of BENOVIĆ et al. [J. Biol. Chem., 262 :9026-9032, (1987)]. This matrix is used to purify *c-myc*-β2AR according to the protocol described by MOUILLAC et al. [J. Biol. Chem., 267 :21733-21737, (1992)]. All the buffers comprise n-dodecyl maltoside (0.05%).

The preparations obtained after affinity chromatography are concentrated using CENTRIPREP and CENTRICON cartridges (AMICON) and the quantity of *c-myc*-β2AR in each sample is determined using [¹²⁵I]-iodocyanopindolol ([¹²⁵I]ICYP) as described by MOUILLAC et al. [J. Biol. Chem., 267 :21733-21737, (1992)]. The preparations of viral particles, of membranes or of β2AR purified by affinity chromatography are subjected to a polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE), under non-reducing conditions, on 10% gel plates. The proteins separated on the gels are transferred onto nitrocellulose and revealed with an anti-*c-myc* mouse monoclonal antibody, and a second anti-mouse antibody coupled to alkaline phosphatase or to horseradish peroxidase. The results are illustrated by Figure 2.

The Western blotting of the cell membrane preparation (Figure 2, lane 1) shows the presence of several immunoreactive bands, between 40 and 50 kDa.

The Western blotting of the preparations of β2AR purified by affinity chromatography (Figure 2, lane 2) shows a single and broad immunoreactive band,

between 46 and 50 kDa, which represents the biologically active mature form of the β 2AR receptor.

The Western blotting of the purified extracellular baculovirus preparation (Figure 2, 5 lane 3) also shows the presence of a single and broad immunoreactive band between 46 and 50 kDa.

These results show that the β 2AR receptor molecules present in the extracellular baculoviruses represent only the biologically active form, unlike the 10 β 2AR receptor molecules present in the cell membrane preparations, which represent a mixture of active and inactive forms.

15 **EXAMPLE 4: PHARMACOLOGICAL PROPERTIES OF VARIOUS RECEPTORS EXPRESSED IN THE EXTRACELLULAR BACULOVIRUSES**

Preparations of extracellular baculoviruses expressing the β 2AR, M1 or D1 receptors are obtained as described in Example 1 above.

The binding of each of the receptors to the 20 ligand is evaluated as described in Example 2 above.

The competitive binding trials in the presence of agonists are carried out using 70 pM of [125 I]ICYP as radioligand. The concentration of the non-labelled ligand varies from 10^{-4} to 10^{-12} M.

25 The saturation assays of the M1-muscarinic (M1-R) and D1-dopaminergic (D1-R) receptors expressed in the viral particles are carried out using 1-100 nM [3H]pirenzepine (NEN, DUPONT) and 0.02-3 nM [125 I]-R(+)-SCH-23390 (NEN, DUPONT) with 5-10 μ g or 1-2 μ g of 30 protein for M1-R and D1-R respectively. To evaluate non-specific binding, 1 μ M atropine (RBI) is added to the reaction mixture for M1-R, and 10 μ M haloperidol (RBI) for D1-R.

The results of these experiments are 35 illustrated by Table I below.

TABLE I

Receptor	Ligand	Kd pM	BMax pmol/mg of protein	Ki μM
β2AR	[¹²⁵ I]ICYP	49.4 ± 11.5		
	Epinephrine			8.98 ± 4.02
	Arterenol			3.27 ± 0.38
M1	[³ H]-Pirenzepine	1360 ± 670	5.56 ± 0.46	
D1	[¹²⁵ I]-SCH23390	118 ± 63	5.21 ± 0.84	

These results show that various receptors of the G-protein-coupled receptor family are expressed in an active form in extracellular baculoviruses.

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EXAMPLE 5: PALMITOYLATION OF THE β2AR RECEPTOR EXPRESSED IN EXTRACELLULAR BACULOVIRUSES

The viral particles expressing c-myc-β2AR are prepared as described in Example 1 above, and the pellet resuspended in PBS. 1 mCi of [³H]palmitate dissolved in dimethyl sulphoxide is added to the viral particles. The reaction is carried out for defined periods in the presence or the absence of 1 μM (final concentration) of isoproterenol.

15

The results are illustrated by Figure 3:

Legend to Figure 3:

☐ : incorporation in the absence of isoproterenol;

☐ : incorporation in the presence of isoproterenol.

20 EXAMPLE 6: COMPARISON OF THE FORMS OF THE β2AR RECEPTOR

The viral particles expressing c-myc-β2AR are prepared as described in Example 1 above, and the pellet resuspended in a buffer (100 mM Tris-HCl, 10 mM MgCl₂, pH 7.4 and protease inhibitors). 1 volume of extracellular baculoviruses and 1 volume of phosphorylation mixture (2.3 μCi/μl of [γ³²P]ATP, 10 mM Tris-HCl, 2 mM MgCl₂, pH 7.4, 25 mM phosphoenol pyruvate, 0.3 mM GTP, 1 mM ATP, 4 U/ml of pyruvate kinase and 20 U/ml of myokinase) are mixed. The

25

reaction is carried out for 25 min at 30°C. At the end of the reaction, the incorporation of ^{32}P is measured in the absence of activator (control) or in the presence of 1 μM of isoproterenol, or of 100 μM of dibutyryl cyclic AMP, or of 100 μM of forskolin. The results are illustrated by Figure 4.

Legend to Figure 4:

On the y-axis: relative incorporation of ^{32}P (arbitrary units)

On the x-axis:

BASAL : control

FRSK : incorporation in the presence of forskolin

cAMP : incorporation in the presence of dibutyryl cyclic AMP

ISO : incorporation in the presence of isoproterenol

EXAMPLE 7: FUNCTIONALITY OF THE $\beta 2\text{AR}$ RECEPTOR EXPRESSED IN EXTRACELLULAR BACULOVIRUSES

The viral particles expressing c-myc- $\beta 2\text{AR}$ are prepared as described in Example 1 above, and the pellet resuspended in a buffer (75 mM Tris-HCl, 12.5 mM MgCl_2 , 2 mM EDTA, pH 7.4 and protease inhibitors). 20 μl of extracellular baculovirus suspension are mixed with 30 μl of reaction medium containing 0.2 mM ATP, 0.090 mM GTP, 0.20 mM cAMP, 0.20 mM isobutylmethylxanthine, 1 μCi [$\gamma^{32}\text{P}$]ATP, 5 mM phosphoenolpyruvate, 0.3 U of pyruvate kinase and 2 U of myokinase. After incubating for 30 min at 37°C, the reactions are stopped by the addition of 1 ml of stop solution (0.4 mM ATP, 0.3 mM cyclic AMP and 25,000 cpm of tritiated cyclic AMP). The activity was determined in the absence of activator (control) or in the presence of one of the following activators: 1 μM of isoproterenol, 10 μM NaF or 100 μM of forskolin. The results are expressed in picomoles of cyclic AMP produced per minute and per milligram of protein. These results are illustrated by Figure 5.

Legent to Figure 5:

On the y-axis: adenylyl cyclase activity (in picomoles of cyclic AMP/min/mg of protein)

On the x-axis:

- 5 BASAL: control
 FRSK: incorporation in the presence of
 forskolin
 NaF: incorporation in the presence of NaF
 ISO: incorporation in the presence of
10 isoproterenol

 These results show that the β 2AR receptor
present in the extracellular baculoviruses is in an
environment which reproduces the natural membrane
environment, and that the extracellular baculovirus
15 preparations can therefore be used in all membrane
receptor applications where a reproduction of this
environment is desirable.